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Analysis of Matrix Protein Gene Nucleotide Sequence Diversity Among Newcastle Disease Virus Isolates Demonstrates that Recent Disease Outbreaks Are Caused by Viruses of Psittacine Origin

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Abstract. Nucleotide sequence analysis was completed for isolates of Newcastle disease virus (NDV; avian paramyxovirus 1) from 1992 outbreaks in cormorants and turkeys. These isolates were of the neurotropic velogenic type. The cormorant and turkey NDV isolates had the fusion protein cleavage sequence ^{109}SRGRRQKR/FVG^{119}, as opposed to the consensus sequence ^{109}SGGRRQKR/FIG^{119} of most known velogenic NDV isolates. The R for G substitution at position 110 may be unique for the cormorant and turkey isolates. For comparative purposes, nucleotide sequencing and analysis of the conserved matrix protein gene coding region were completed for isolates representing all pathotypes. Phylogenetic relationships demonstrated that there are two major groups of NDV isolates. One group includes viruses found in North America and worldwide, such as B1, LaSota, Texas/GB, and Beaudette/C. The second group contains isolates, such as Ulster/2C, Australia/Victoria, and Herts/33, considered exotic to North America. Within this second group are viruses of psittacine origin. The viruses from 1992 outbreaks of Newcastle disease in North America, and an isolate thought to have caused the major outbreak in southern California during the 1970s, are most closely related to an NDV isolate of psittacine origin.

Key words: avian paramyxovirus, reverse transcription, polymerase chain reaction, molecular cloning, phylogenetics, pathotype

Introduction

Newcastle disease virus (NDV), designated avian paramyxovirus-1 in the family Paramyxoviridae, has a single-strand, negative-sense genomic RNA of approximately 15 kb. The genome codes for six viral proteins, including an RNA-directed RNA polymerase (L), hemagglutinin-neuraminidase (HN) protein, fusion (F) protein, matrix (M) protein, phosphoprotein (P), and nucleocapsid (NP) protein (1). The enveloped virus has a wide host range, with members from all orders of birds reported to be susceptible to infection (2). Outbreaks of Newcastle disease were first reported from Java, Indonesia, and Newcastle-upon-Tyne, England in 1926 (3,4), and the disease now has a worldwide distribution (1). Isolates of NDV are categorized into three pathotypes, depending on the severity of disease caused by an isolate (5). Lentogenic NDV isolates do not usually cause disease in adult birds and are considered of low virulence. Viruses of intermediate virulence that cause respiratory disease, but not usually fatal, are termed *mesogenic*.

Among the highly virulent velogenic NDV isolates, the viscerotropic and neurotropic forms are found worldwide (1). Viscerotropic velogenic viruses have entered the United States via importation of psittacine birds (6–8). A viscero-

tropic velogenic virus of psittacine origin was thought to be the causal agent of a major Newcastle disease outbreak in southern California poultry during the early 1970s (9,10). Outbreaks of Newcastle disease in cormorants in the north-central United States and south-central Canada (11,12), as well as in a commercial North Dakota turkey flock during 1992, have been attributed to neurotropic velogenic viruses (National Veterinary Services Laboratory, APHIS, USDA, personal communication).

The molecular determinants of NDV virulence are primarily dependent upon the amino acid sequence of the F protein cleavage site (13–15) and the ability of specific cellular proteases to cleave the F proteins of different pathotypes (16,17). Fewer basic amino acids are present in the F protein cleavage site of lentogenic NDV isolates than in either mesogenic or velogenic strains (14). Differences in the size of the HN protein have also been detected, with avirulent lentogenic viruses encoding a larger precursor (18,19), which in some isolates must be cleaved for activation (19). The size differences are due to point mutations in the gene, resulting in conversion of stop codons to sense codons, thus extending the C-terminus of the protein. In virulent NDV isolates encoding a smaller protein, the C-terminal encoding regions of the HN gene are thought to be analogous to pseudogenes (19). Sequence analysis of the HN protein (19) and F protein (20) genes of 11 different NDV isolates revealed that there are three possible evolutionary lineages. These lineages apparently evolved by accumulating varying numbers of point mutations and not by recombination (19,20).

Phylogenetic analysis of viral genomic and protein sequences is now a common method used to determine origins and evolutionary relationships among isolates (reviewed in 21). Among mumps virus isolates, another paramyxovirus, the M gene, is highly conserved compared with other viral genes, and the sequence is not greatly affected during virus attenuation (22). Molecular epidemiology has been used to determine relationships among measles virus isolates during recent outbreaks of the disease. Matrix protein gene evolutionary relationships were the same as relationships based upon the NP gene, even though fewer changes occurred in the M protein gene (23). Consequently, the M protein gene was used to determine phylogenetic relationships among recently isolated virulent North American NDV isolates as compared with previously reported viruses.

Materials and Methods

Virus Isolates and Histories

Pathogenic (neurotropic velogenic) NDV isolates were obtained from cormorants in Michigan (C-MI) and Minnesota (C-MN) during outbreaks of Newcastle disease in 1992 (11,12; originally provided by the National Wildlife Health Research Center, Madison, WI). Subsequently, a neurotropic velogenic NDV was isolated from turkeys (turkey/ND) in an unvaccinated commercial North Dakota flock with Newcastle disease (provided by the Diagnostic Virology Laboratory, NVSL, APHIS, USDA, Ames, IA). A lentogenic vaccine virus, VGGA (provided by P. Villegas, Poultry Diagnostic Research Center, College of Veterinary Medicine, University of Georgia), a viscerotropic velogenic isolate from psittacines (Largo) (24), and a viscerotropic NDV isolated during the 1972–1974 outbreak of Newcastle disease in California (Fontana) (10) were compared with other reference NDV isolates (all four pathotypes). Strains B1 (25), LaSota (25), Ulster/2C (18,28), and Queensland/V4 (29) are lentogenic vaccine viruses. The D26 virus is a previously characterized lentogenic NDV from ducks in Japan (30). Kimber is a mesogen of intermediate virulence (25), while Texas/GB (25,31), Beaudette/C (32–34), and Australia/Victoria (35–37) are neurotropic velogens. Herts/33 is a viscerotropic velogen (38). All viruses examined are listed in Table 1.

Initial characterization of all viral isolates was accomplished by hemagglutination inhibition (HI) with NDV-specific polyclonal antisera (5). Further differentiation of individual isolates was completed using HI with a series of monoclonal antibodies developed for clinical use (39). In some instances, virulence of NDV isolates was assessed (5) by mean death time in eggs (MDT),

Table 1. Virus isolates examined for phylogenetic analysis of the matrix protein gene nucleotide and predicted amino acid sequence

Virus	F sequence[a]	Pathotype[b]
B1	SGGGRQGR LIG	L
La Sota	SGGGRQGR LIG	L
VG/GA	SGGGRQGR LIG	L
D26	SGGGKQGR LIG	L
Ulster/2C	SGGGKQGR LIG	L
Queensland/V4	SGGGKQGR LIG	L
Kimber	SGGRRQKR FIG	M
Texas/GB	SGGRRQKR FIG	NV
Austrl./Victoria	SGGRRQKR FIG	NV
Beaudette/C	SGGRRQKR FIG	NV
Herts/33	SGGRRQRR FIG	VV
Largo	SGGRRQKR FVG	VV
Fontana	SGGRRQKR FVG	VV
Turkey/ND	SRGRRQKR FVG	NV

[a] Amino acids represent the cleavage site rom residues 109 to 119 of the fusion protein. The F2 portion is on the N-terminal side, with the F1 on the C-terminal portion of the space representing cleavage. The partial amino acid sequence was derived from the predicted amino acid sequence of a 254 base-pair reverse-transcription polymerase chain reaction product specific for Newcastle disease virus genomic RNA.
[b] Pathotype is listed as L for lentogenic, M for mesogenic, NV as neurotropic velogenic, and VV for viscerotropic velogenic. Standard pathotype tests were conducted or listed as reported.

by intracerebral pathogenicity index (ICPI), and by intracloacal inoculation.

Virus Replication, RNA Purification, and Reverse Transcription–Polymerase Chain Reaction

Isolates of NDV were replicated in embryonated eggs (5) and purified by sucrose gradient centrifugation (13), after which genomic RNA was acid-guanidinium-phenol extracted (40). In some cases viral RNA was extracted from infectious egg allantoic fluid. Degenerate oligonucleotide primers were obtained by alignment of published NDV nucleotide sequences and analysis of these data using PRIMER2™ (Scientific & Educational Software, Stateline, PA). Reverse transcription–polymerase chain reaction (RT-PCR) using genomic NDV RNA as a template was completed essentially as described (41), except that initial reverse transcription was conducted using Superscript™ (Life Technologies, Gaithersburg, MD) at 45°C (42).

Oligonucleotide Primers, Nucleotide Sequencing, and Analysis

Resultant RT-PCR products were purified using Microcon™ columns and were spectrophotometrically quantitated. Direct nucleotide sequencing was completed using Taq polymerase with RT-PCR primers, internally derived primers, and an automated nucleic acid sequencer (43,44). The RT-PCR primers were designed to amplify regions of the F protein gene, including the fusion cleavage site (19), and the entire M protein gene (30,34,37). Amplification products were also directly cloned (45) for nucleotide sequencing (44). Nucleotide sequence analysis was conducted using the IntelliGenetics GeneWorks 2.45™ (Mt. View, CA) with UPGMA (46), PAUP (47), and MEGA (48) software. All oligonucleotide primer sequences used during these investigations are available from the author upon request. GenBank accession numbers for the M protein gene sequences reported are U25828–U25838.

Results

Predicted Amino Acid Sequence of the Fusion Protein Cleavage Site Among NDV Isolates

The F protein cleavage site sequences and pathotypes of the 14 NDV isolates examined during this investigation are presented in Table 1. All pathotypes and many geographic origins are represented. The fusion cleavage site amino acid sequence was ^{109}SGGGRQGR/LIG^{119} in North American lentogenic isolates B1, LaSota, and VGGA. However, the lentogenic isolates—D26 from Japan, Ulster/2C from northern Ireland, and the Australian Queensland/V4—had a K for R substitution at position 113 in the F-protein amino acid sequence. A mesogenic isolate from North America, Kimber, had the F protein cleavage site amino acid sequence ^{109}SGGRRQKR/FIG^{119} common to virulent NDV isolates. This sequence was shared by the neurotropic velogenic Texas/GB and Beaudette/C isolates from

North America. A neurotropic velogenic isolate, Australia/Victoria, and the viscerotropic velogenic Herts/33 from the United Kingdom also share a F protein cleavage site sequence common to virulent NDV.

Three velogenic isolates had amino acid differences in the F-protein cleavage sequence from the other previously known velogenic viruses. Two viscerotropic velogenic viruses, one isolated from psittacines in North America (Largo) and another virus isolated from chickens thought to be the causal agent of the southern California outbreak (Fontana), had the sequence ^{109}SGGRRQKR/FVG^{119} at the F-protein cleavage site. The turkey/ND isolate has a fusion protein cleavage sequence identical to the Largo and Fontana isolates, with the exception of an R for G difference at position 110. This sequence is identical to that of the cormorant/MN isolate (data not shown). The V for I substitution at residue 118 is common to these three NDV isolates.

Nucleotide Sequence of the NDV Matrix Protein Genes and Alignment of the Predicted Amino Acid Sequences

At the nucleotide sequence level, 759 residues of the 1223 nucleotides of the M protein gene amplification product are shared among all isolates, for a sequence similarity of 62%. A total of 247 of the 364 predicted amino acids are shared by the M proteins, for a 68% sequence similarity among all the isolates examined. Within the M protein predicted amino acid sequence, the Largo, Fontana, turkey/ND, and Herts/33 shared conserved differences at positions 16, 193, 211, 217, 243, 247, 263, 327, and 356 with Ulster/2C, D26 and Australia/Victoria as compared with the LaSota, Beaudette/C, B1, Texas/GB, Kimber, and VGGA isolates (Fig. 1). At residue 38, a T for A substitution occurs, along with an R for K substitution at position 188 in the M protein sequence of the Largo, Fontana, turkey/ND, and Herts/33 isolates as compared with other NDV isolates. The M for R substitution at position 142 is unique to the Largo psittacine isolate, Fontana, and the turkey/ND viruses. At position 131, an I for V difference is shared by the Largo, turkey/ND, and Herts/33 viruses relative to the other isolates. Other amino acid differences unique to these isolates are found at residues 19, 83, and 265. A virus obtained from infected cormorants during the 1992 Newcastle disease outbreak in Minnesota (cormorant/MN) had the same nucleotide and amino acid sequence as the turkey/ND isolate (data not shown).

Phylogenetic Analysis of the Matrix Protein Gene Among NDV Isolates

Genetic distances calculated from the number of nucleotide differences in the M protein gene between individual isolates indicates that there are two groups of NDV isolates (Table 2). One group includes isolates B1, Beaudette/C, LaSota, Kimber, VGGA, and Texas/GB. These viruses are greater than 95% similar at the nucleotide sequence level of the M protein gene when compared with B1 as a reference. Within this group the lentogenic B1 vaccine isolate has the fewest nucleotide sequence differences with the other lentogenic vaccine viruses LaSota and VGGA. Fewer numbers of sequence differences are shared with the mesogenic Kimber isolate compared with the neurotropic velogenic Beaudette/C and Texas/GB isolates relative to the lentogenic strains.

Based on nucleotide sequence differences within the M-protein gene, a second group includes isolates Largo, turkey/ND, Fontana, Australia/Victoria, Herts/33, D26, Ulster, and Queensland/V4 (Table 2). These viruses are 91% or less similar to the B1 virus at the nucleotide sequence level of the M-protein gene following pairwise comparisons. Within this second group, the lentogenic viruses D26, Ulster, and Queensland/V4 are most closely related. The viscerotropic velogenic psittacine Largo isolate is most similar in nucleotide sequence to the neurotropic turkey/ND and viscerotropic Fontana isolates. Within this second group, the viscerotropic velogenic Herts/33 virus has fewer sequence differences from the neurotropic Australia/Victoria virus relative to the other isolates.

When determining phylogenetic relationships among NDV isolates (Fig. 2), two major groups arise containing the same viruses as analyzed by

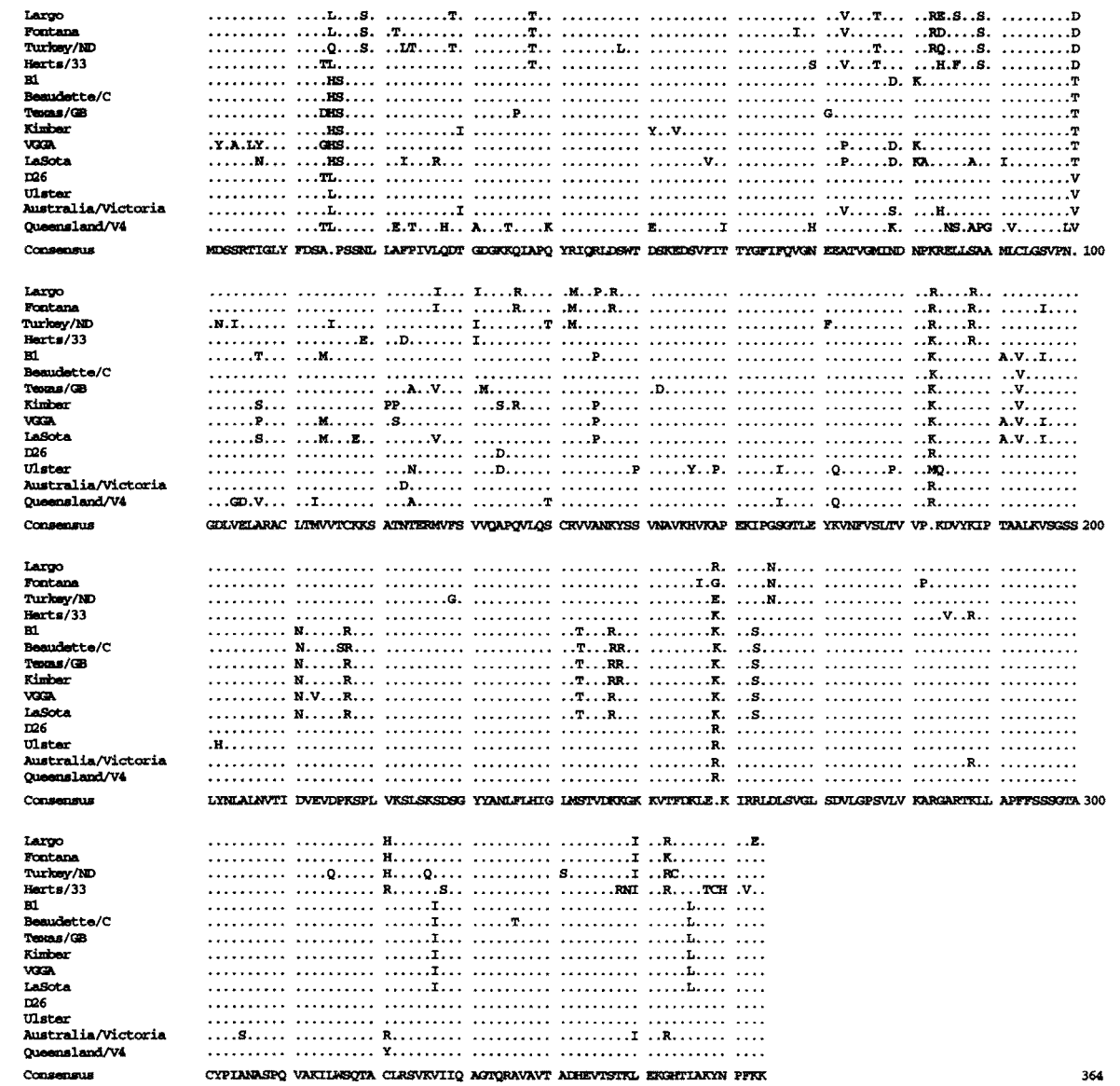

Fig. 1. Predicted amino acid sequence alignment of the matrix proteins for Newcastle disease virus isolates. Virus isolates are listed in Table 1, with their respective histories described in the text. Using NDV matrix protein gene–specific primers, a 1223 base-pair product was obtained following RT-PCR using NDV genomic RNA as a template for all isolates. The RT-PCR product was sequenced using oligonucleotide primers interior to the gene. Data for the Australia/Victoria (37), D26 (30), and Beaudette/C (34) isolates were from published sequences available in GenBank.

pairwise distances between isolates. Similar results are obtained using either the M-protein gene nucleotide sequences or the predicted amino acid sequences. The lentogenic B1, VGGA, and LaSota isolates appear more closely related to each other, with a clear demarcation between them and the more virulent Kimber, Beaudette/C, and Texas/GB isolates among group B viruses. Within the A group, lentogenic D26, Ulster, and Queensland/V4 viruses separate from the virulent isolates. The viscerotropic velogenic Fontana isolate is most closely related to the viscerotropic psittacine Largo and neurotropic turkey/ND virus. The viscerotropic velo-

Table 2. Pairwise distances between Newcastle disease virus isolates calculated from the matrix protein gene nucleotide coding sequence information[a]

	B1	BeC	LS	Kim	VG	GB	Larg	ND	Font	AV	H33	D26	Ulst	V4
B1	—	.024	.014	.040	.020	.038	.155	.169	.148	.113	.141	.097	.102	.124
BeC	29	—	.034	.025	.040	.022	.155	.165	.150	.109	.137	.096	.096	.121
LS	17	41	—	.046	.024	.041	.162	.176	.158	.122	.151	.106	.110	.129
Kim	49	30	56	—	.053	.038	.164	.176	.158	.120	.146	.111	.111	.134
VG	25	49	29	65	—	.048	.168	.179	.164	.126	.155	.114	.118	.138
GB	46	27	50	46	59	—	.163	.175	.158	.122	.147	.107	.106	.128
Larg	189	188	198	200	205	199	—	.068	.092	.106	.119	.127	.133	.143
ND	207	201	215	215	219	214	83	—	.128	.133	.138	.148	.155	.164
Font	181	182	193	193	200	193	113	157	—	.100	.133	.127	.137	.148
AV	134	129	144	142	149	145	125	157	118	—	.086	.084	.087	.109
H33	172	166	184	178	190	180	145	169	163	102	—	.111	.115	.133
D26	119	117	130	136	139	131	155	181	155	100	136	—	.048	.036
Ulst	125	117	135	136	144	130	163	190	167	103	141	59	—	.074
V4	151	147	158	164	169	157	175	201	181	129	163	44	90	—

[a]Pairwise distances are based on the number of nucleotide sequence differences in the matrix protein gene between isolates. Above the diagonal is the mean distance, expressed as the reciprocal, and absolute differences are presented below. Abbreviations of isolates are as follows with year of isolation: B1: Hitchner/B1, 1947; BeC: Beaudette/C, 1945; LS: LaSota, 1946; Kim: Kimber, 1947; VG: VGGA, 1992; GB: Texas/GB, 1948; Larg: Largo, 1974; ND: Turkey/ND, 1992; Font: Fontana, 1972; AV: Australia/Victoria, 1967; H33: Herts/33, 1933; D26: D26, 1976; Ulst: Ulster/2C, 1967; V4: Queensland/V4, 1967.

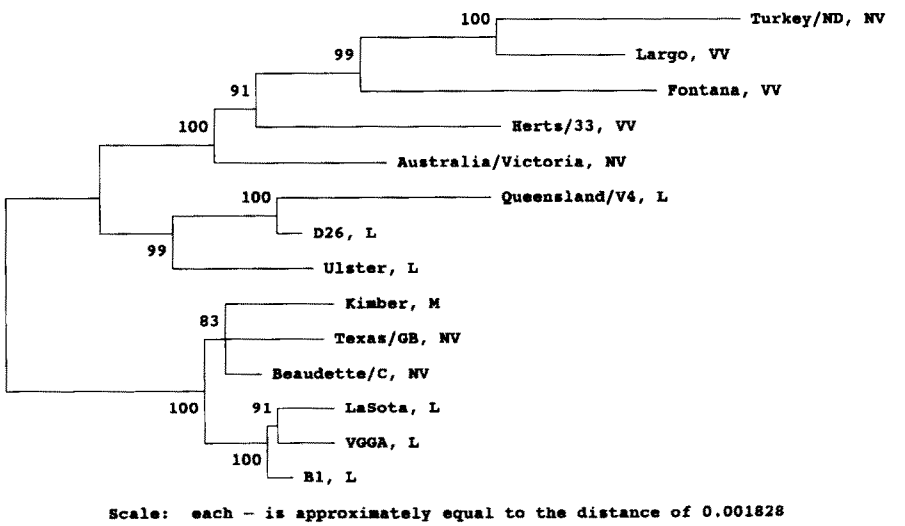

Fig. 2. Phylogenetic relationships among Newcastle disease virus isolates based on matrix protein gene nucleotide-coding sequences. Virus isolates are listed in Table 1, with their respective histories described in the text. Nucleotide sequence alignment was completed, and an unrooted neighbor-joining tree was constructed based on Jukes-Cantor distances followed by bootstrap analysis with 2000 replicates. Bootstrap confidence limits are listed for branch points as a percentage. Pathotype is listed as L for lentogenic, M for mesogenic, NV as neurotropic velogenic, and VV for viscerotropic velogenic.

genic Herts/33 and neurotropic Australia/Victoria viruses in this group are related most closely to each other. The same topology exists among isolates using parsimony methods, such as PAUP, to construct phylogenetic trees (data not shown). Also, using an outgroup in these analyses, such as measles or mumps virus M-protein amino acid or genomic nucleotide sequences, does not affect the phylogenetic relationships among NDV isolates.

Discussion

During a recent outbreak of Newcastle disease involving cormorants in the north-central United States and south-central Canada (11,12), an unvaccinated commercial turkey flock became infected. The viruses isolated from both cormorants and turkeys were neurotropic velogenic NDV, with the same monoclonal antibody reactivities based on hemagglutination inhibition (unpublished). The 1992 turkey/ND and cormorant NDV isolates had an F-protein cleavage site amino acid sequence required for velogenic viruses that closely resembles that of highly virulent viruses isolated from chickens during an outbreak of Newcastle disease in the Republic of Ireland (49,50). However, a V for I substitution at position 118 of the turkey/ND F protein was shared with a psittacine isolate (24) and a virus isolated during the major Newcastle disease outbreak during the early 1970s in southern California (10). This amino acid substitution at residue 118 in the F-protein cleavage site is similar to what was reported for certain waterfowl NDV isolates in Europe (50). At residue 110 of the F protein, an R for G substitution was detected that was unique to the turkey/ND and cormorant/MN NDV isolates.

Matrix proteins of paramyxoviruses are considered to be relatively conserved among isolates of different virus types (22,23). This is substantiated by the low 0.12 ratio of nonsynonymous to synonymous base changes using Jukes-Cantor proportional differences (48). Although this method may give a low estimate, it still reflects a highly conserved gene not subject to much selection pressure (51). Despite this, differences in monoclonal antibody reactivities to the M protein do occur among NDV isolates (52). Consequently, the M-protein genes of several NDV isolates, in addition to the Australia/Victoria (37), Beaudette/C (34), and D26 (30) viruses previously reported, were sequenced, and their genomic nucleotide and predicted amino acid sequences were determined. Several differences in the amino acid sequence of the predicted M proteins occurred among various isolates that corresponded to genotypic similarities. This included an R for S substitution at position 263, previously determined to be essential for NDV matrix protein nuclear localization (53).

Phylogenetic analysis of the M-protein gene and predicted amino acid sequences indicate there are two major groups of NDV isolates. Using simple distance matrix methods, such as UPGMA, the three original subtypes previously reported (19,20) do not distinctly separate. Both neighbor-joining and parsimony techniques produce the same basic phylogenetic topology among NDV isolates as depicted in Fig. 2, which is similar to previous findings (19,20). Interestingly, the avirulent lentogens may be separated from their respective mesogenic and velogenic counterparts within their respective groups.

Velogenic NDV can be isolated from psittacine birds imported into the United States (6–8), and outbreaks in psittacines may still occur (7). Also, a virus causing the last major outbreak of Newcastle disease in the United States during the early 1970s in southern California was epidemiologically linked to a virus of pet bird origin (9). The phylogenetic relationships reported here indicate that these psittacine-type viruses may be circulating in wild and pet bird populations, or are being introduced from sources outside the United States.

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